

[RAPID COMMUNICATION]

RAPD analysis: An Efficient Method of DNA Fingerprinting in Fishes

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ABSTRACT—Random Amplified Polymorphic DNA (RAPD) generated using arbitrary primers of 9, 10, 16 and 20 nucleotide lengths by Polymerase Chain Reaction (PCR) was investigated in 12 species of fishes. We found that the amplification products were best resolved by Urea-SDS-PAGE and detected by silver staining. The amplification products ranged from 25 to 75 depending on the primer and template combination. The random primers generated unique fingerprints for each species of fish in terms of number and position of RAPDs. Our results showed that the fish species can be distinguished from each other by RAPDs. The complexity of the RAPDs in the fingerprints may be manipulated to suit the requirement of the study. The use of RAPD in taxonomy, fishery management and fish culture is discussed.

INTRODUCTION

The conventional DNA fingerprinting method, involving restriction fragment length polymorphism (RFLP) assay, requires large quantities of relatively pure DNA, specific DNA probes and generally uses short-lived radio-isotopes in the detection system. It is also laborious and time consuming making it impractical for large population based studies. Fingerprinting by polymerase chain reaction (PCR) technique requires much less DNA but a major limitation is the requirement of

target DNA-sequence information for designing specific primers. Recently, Williams *et al.* [18] and Welsh and McClelland [17] described a novel PCR based method termed Random Amplified Polymorphic DNA (RAPD) fingerprinting. This technique allows detection of DNA polymorphisms by randomly amplifying multiple regions of the genome by PCR using single arbitrary primers designed independent of target DNA sequence. Since the RAPD technique involves enzymatic amplification of target DNA by PCR using arbitrary primers it is also called Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) or DNA Amplification Fingerprinting (DAF). This method overcomes some technical limitations of the earlier fingerprinting methods and has wide applications including: genetic fingerprinting of bacteria, plants, few animal species and humans [1-3, 6, 17, 18]; creating linkage maps [16]; locating disease resistance genes [11, 12, 14]; and identifying chromosome-specific markers [15]. AP-PCR has been used for the detection of DNA polymorphisms of few fish species including colour mutants of tiger barb, *Barbus tetrazona* and guppy, *Poecilia reticulata* [4, 5]. Kubota *et al.* [10] used this method for the detection of radiation induced DNA damages in Japanese medaka fish, *Oryzias latipes*. Hence, RAPD fingerprinting technique is robust, simple, fast, sensitive and particularly suited to problems where the genome is anony-

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mous or quantity of genomic DNA available is limited. It can also be used for analyses of museum specimens and rare fishes using DNA isolated from scales and clipped fins without destroying the whole organism.

We used this technique for generating DNA fingerprints in 10 species of tropical and two species of temperate fishes representing seven families, *viz.*, Belontiidae (*Betta splendens*), Anabantidae (*Colisa lalia*, *Trichogaster microlepis*), Cyprinidae (*Cyprinus carpio*, *B. tetrazona*, *Brachydanio rerio*), Poeciliidae (*P. reticulata*, *Xiphophorus maculatus*), Cichlidae (*Oreochromis niloticus*), Characidae (*Hyphessobrycon innesi*) and Salmonidae (*Onchorhynchus nerka*, *Salmo salar*). We report here the usefulness of this method in combination with Urea-SDS-PAGE in generating RAPDs among the fishes.

MATERIALS AND METHODS

Extraction of genomic DNA: Tissues from individual fishes were pulverized after flash freezing in liquid nitrogen, incubated with mild shaking at 55°C in 10 volumes of extraction buffer (50 mM Tris-Cl, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM NaCl; 100 mM DTT; 1.0% SDS; 0.5 mg/ml proteinase K). After phenol extraction and ethanol precipitation, DNA was dried using Speed Vac concentrator (Savant), resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0) and stored at 4°C.

Oligonucleotide Primers: Random primers synthesized on ABI 394 DNA synthesizer and purified by an ABI oligonucleotide purification cartridge were purchased from the Bioprocessing Technology Unit, National University of Singapore. The 10 primers used were of 9–20 nucleotides (9-to 20-mer) in length with G+C content ranging from 45.0–77.7%. The melting temperature (T_m) values of the primers ranged from 28°C to 58°C. The details of the primers are given in Table 1.

DNA Amplification: Samples were amplified in 50 μ l reaction mixtures containing 1X PCR buffer (Promega), 15 mM MgCl₂, 1.0 M primer, 0.3 to 0.5 μ g of genomic DNA, 2 mM of each dNTP (Promega) and 2.0 units of Taq DNA polymerase (Promega). The total reaction mix was overlaid with 40 μ l of mineral oil (Sigma). Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR System 9600 programmed for 30 cycles of 3 min denaturation at 94°C, 3 min low stringency annealing at 37°C and 2 min primer extension at 72°C. At the end a final extension for 10 min was performed at 72°C.

Denaturing polyacrylamide gel electrophoresis: Amplified fragments were separated by SDS-PAGE (3% stacking and 8% resolving gel) in a Mini-Protean II (Bio-Rad; Fig. 2) or Urea SDS-PAGE (3% stacking and 8% resolving gel containing 7 M urea) in Sturdier SE 400 (Hoefer) Electrophoresis unit. Eight to ten μ l of each PCR

TABLE 1. Details of Arbitrary Primers

Name	Nucleotide length	Sequence (5'-3')	(G+C)%	T_m (°C)
NUSZG1	9-mer	TTGCGTCCA	55.5	28
NUSZG2	9-mer	GCACTGTCT	55.5	28
NUSZG3	9-mer	GGTAACGCC	66.6	30
NUSZG4	9-mer	GGAGCTGGC	77.7	32
NUSZG5	10-mer	AGGTCCTGA	50.0	30
NUSZG6	10-mer	CGGTCCTGT	60.0	32
NUSZG7	10-mer	AATCGGGTCG	60.0	32
NUSZG8	10-mer	TGCCGAGCTG	70.0	34
NUSZG9	16-mer	TGCCTGTGGGGAATCC	62.5	52
NUSZG10	20-mer	TATGTAAAACGACGGCCAGT	45.0	58

product was loaded onto polyacrylamide slab gels of 0.75 mm (7×8 cm; Mini-Protean II) or 1.00 mm (14×18 cm; Sturdier SE 400) thickness. The electrode buffer (pH 8.3) contained 0.025 M Tris-Cl, 0.2 M glycine and 0.1% SDS. The samples were loaded together with a PCR dense dye containing 50 mM EDTA, 30% glycerol, 0.25% xylene cyanol FF and 0.25% bromophenol blue. Electrophoresis was carried at 100 V for 3¼ hr in Mini-Protean or 16 hr in Sturdier gel unit.

Silver staining: Silver staining method of Herring *et al.* [8] was used with slight modifications to stain the gels. The gels were fixed with 10% ethanol-0.5% acetic acid for 1 hr and then soaked in 0.011 M silver nitrate for 30 min. After rinsing the gels twice briefly in distilled water, the reduction reaction was carried out for a maximum of 10 min with a solution of 0.75 M sodium hydroxide and 0.085 M formaldehyde till the bands were clearly visible. The reaction was stopped by transferring the gels to 0.07 M sodium carbonate for 30 min. Prior to drying, the gels were photographed with transmitted light using a Nikon F-501 camera fitted with a 55 mm f2.8 Micro-Nikkor lens and Kodak TMAX 100 black and white film. Bio-Rad gel dryer 583 was used to dry the gels after briefly soaking in a solution of 30% methanol and 5% glycerol.

RESULTS AND DISCUSSION

Random Amplified Polymorphic DNA (RAPD) in 12 species of fishes was investigated. Both the short (9 and 10 nucleotide) and medium length (16 and 20 nucleotide) primers generated discrete DNA amplified fragments of varying lengths and revealed RAPD variation among the species. We found that addition of 7 M urea to the 8% resolving gels in SDS-PAGE improved the amplified fragment separation considerably. Generally the fragments of range 100 to 3500 bp could be adequately resolved by Urea-SDS-PAGE. The spectrum of amplified products was reproducible for a particular template-primer combination. The technique of DNA amplified fragment separation and detection system play important roles in RAPD analysis. For the purpose of comparison

the RAPD patterns obtained using similar electrophoretic conditions, gel size and detection system should be used.

The RAPD profiles were found to be similar in terms of mobility and intensity of the bands for different individuals of the same species as seen in our example of the guppy, indicating specificity of the DNA patterns for a given species (Fig. 1). Among the 10 guppies (5 male and 5 female full-sibs obtained from a single-pair mating) tested, they shared many monomorphic (constant) bands with few polymorphic (variable) bands accounting for individual differences (Fig. 1). Hence, these fingerprints indicated low genetic variability which could be due to inbreeding, as the parents of the individuals tested were from a small random breeding stock of about 50 in number maintained for several years in our laboratory. The presence of simple repetitive male specific DNA revealed by oligonucleotide fingerprinting has been reported in the guppy, a species with XX/XY sex determining mechanism [13]. We did not find any sex specific RAPD markers for male or female guppies using the 16-mer RAPD primer

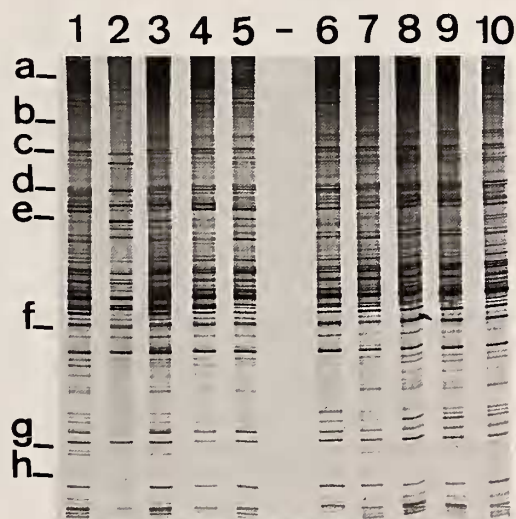


FIG. 1. RAPD fingerprints generated by 16-mer (NUSZG9) in 5 male (lanes 1-5) and 5 female (lanes 6-10) individuals of the guppy, *Poecilia reticulata*. Lane '-' is of negative reactions without template DNA. Fragment sizes (bp) of lambda DNA-BstE II digest molecular weight standard: a) 3675, b) 2323, c) 1929, d) 1371, e) 1264, f) 702, g) 224, h) 117.

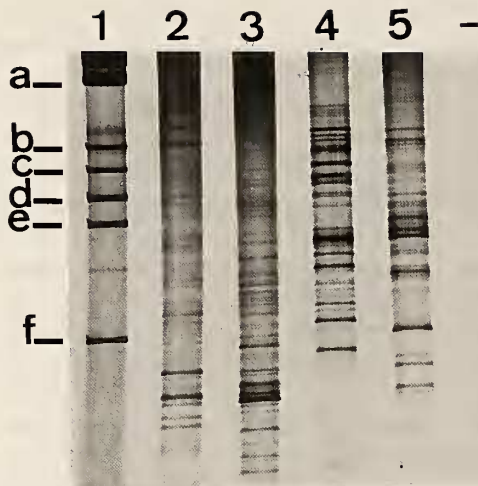


Fig. 2. DNA profiles from a single tiger barb, *Barbus tetrazona* obtained by using four primers of different nucleotide sequences and lengths. DNA amplification products by: 9-mer, NUSZG4 (lane 2); 10-mer, NUSZG8 (lane 3); 16-mer, NUSZG9 (lane 4); and 20-mer, NUSZG10 (lane 5). The first and last lanes are of lambda DNA-BstE II digest and negative reactions without template, respectively. RAPD bands resolved by SDS-PAGE in Mini-Protean II (BIO-RAD).

(NUSZG9) (Fig. 1).

Figure 2 showed that four primers of different sequences and lengths generated different profiles in a single individual tiger barb. Also, the DNA profiles of 10 individual fishes representing 10 species (lanes 1-10) in Fig. 3 (9-mer, NUSZG3), Fig. 4 (10-mer, NUSZG5) and Fig. 5 (20-mer, NUSZG10) show clearly that different patterns of amplified products are generated by different primers. We have found that the primers of same length but with different sequences generated different DNA patterns in a single fish [4, 5]. These results showed that this technique can be manipulated by changing the primer sequence and length to generate amplification products of desired complexity to suit different purposes like genetic mapping or genotyping [1]. Depending on the particular primer and template combination, the number of RAPD ranged from 25 to 75. Each primer detected an average of 30 RAPD variable bands among the 12 species of fishes studied. However, there was no clear relation between the

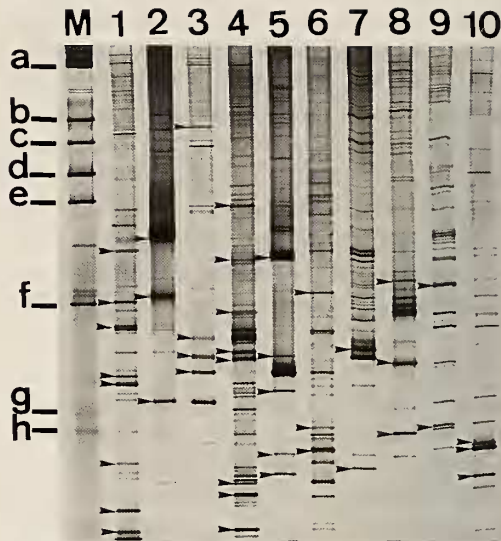


Fig. 3. Amplification fingerprints in 10 species of tropical fishes generated by a 9-mer, NUSZG3. Lane M: Molecular weight marker is lambda DNA-BstE II digest. Lane 1: *Betta splendens*; Lane 2: *Colisa lalia*; Lane 3: *Trichogaster microlepis*; Lane 4: *Cyprius carpio*; Lane 5: *Barbus tetrazona*; Lane 6: *Brachydanio rerio*; Lane 7: *Poecilia reticulata*; Lane 8: *Xiphophorus maculatus*; Lane 9: *Oreochromis niloticus*; Lane 10: *Hyphessobrycon innesi*. Arrows indicate RAPD variable bands among the different species.

length of the primers and the number of amplified fragments generated. The number of amplified products may be related to the G+C content of primer and template DNA sequence rather than to the primer length [1]. G+C content of the primers used in our study ranged from 45.0-77.7%. Figure 2 shows some evidence that primers of higher G+C content generate more amplified products.

The 9-mer (NUSZG3) generated a total of about 40 clearly noticeable RAPD variable bands among the 10 species of tropical freshwater fishes (Fig. 3), while the 10-mer (NUSZG5) (Fig. 4), 16-mer (NUSZG9) and 20-mer (NUSZG10) (Fig. 5) generated 31, 20 and 28 RAPD variable bands, respectively, among the 12 species of fishes which included two cold water species. The fingerprints generated by the four different primers revealed unique profiles for each species in terms of number and position of RAPD bands. Thus, the RAPD generated for each species can be efficiently used

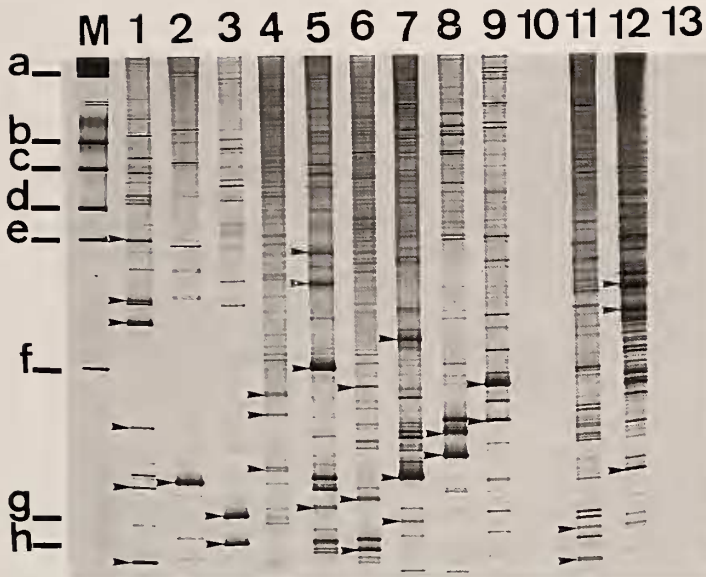


FIG. 4. DNA fingerprints generated by a decamer primer, NUSZG5 in 12 species of fishes. Lanes M and 1 to 10 are as in Fig. 3. Lane 11: *Onchorhynchus nerka*; Lane 12: *Salmo salar* and Lane 13: control reactions without template DNA.

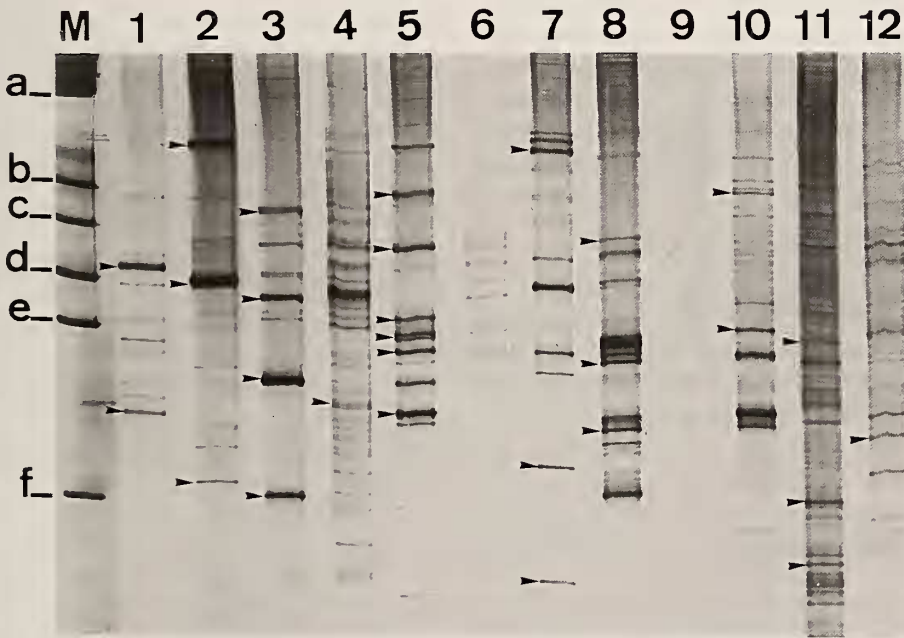


FIG. 5. Fingerprints generated by the 20-mer (NUSZG10) in 12 species of fishes. Lanes M and 1-12 are as in Fig. 4.

as supporting markers for taxonomic identification. Further confirmation can be achieved by considering the RAPD markers generated by more than one primer for the same species. In taxonomy

and systematics, species-specific RAPD markers could be an invaluable tool for species verification and in establishing the status of organisms of controversial systematics. RAPDs that are di-

agnostic at different taxonomic levels can be generated by employing different primers and they can be used to determine the relatedness between taxa for which diagnostic RAPD fingerprints have been established [7]. However, for RAPD fingerprinting to provide supporting evidence for taxonomic relationships in fishes, data should be generated using adequate sample size for each species. RAPD markers can play an important role in fishery management and conservation genetics such as studying the genetic effects of mixing cultured fish with wild populations. Such DNA markers could also be used to assess the impact of intentional or accidental release of farmed fishes to the wild [9]. From the point of view of fish culture, RAPD methodology has been shown to be useful in preliminary pedigree analyses [5] and in the detection of phenotypic-specific DNA polymorphic markers in different colour mutants of two freshwater aquarium fishes [4, 5].

Our results show that RAPD can be used to generate useful fingerprints characteristic of fish species and for genotyping individuals within the species. Thus, it provides an efficient and sensitive method which can be used to estimate genetic variability, relatedness, inbreeding levels, species/strain verification, pedigree analyses, detection of economic traits and in other marker-based studies in fishes.

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